Label-Retaining Cells Reside in the Bulge Area of Pilosebaceous Unit: Implications for Follicular Stem Cells, Hair Cycle, and Skin Carcinogenesis

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Summary

Inconsistent with the view that hair follicle stem cells reside in the matrix area of the hair bulb, we found that label-retaining cells exist exclusively in the bulge area of the mouse hair follicle. The bulge consists of a subpopulation of outer root sheath cells located in the midportion of the follicle at the arrector pili muscle attachment site. Keratinocytes in the bulge area are relatively undifferentiated ultrastructurally. They are normally slow cycling, but can be stimulated to proliferate transiently by TPA. Located in a well-protected and nourished environment, these cells mark the lower end of the "permanent" portion of the follicle. Our findings, plus a reevaluation of the literature, suggest that follicular stem cells reside in the bulge region, instead of the lower bulb. This new view provides insights into hair cycle control and the possible involvement of hair follicle stem cells in skin carcinogenesis.

Introduction

The bulbar region of the hair follicle contains a pool of relatively undifferentiated epithelial cells, termed matrix cells, which give rise to the hair and its surrounding inner root sheath (Hashimoto and Shibazaki, 1976). During the growing phase (anagen) of the hair cycle, these matrix cells proliferate extremely rapidly with a doubling time of 18–24 hr (Van Scott et al., 1963). This proliferation appears to be tightly controlled. Not only does it depend on an adjacent population of specialized mesenchymal cells, which form the dermal papilla, but it is also cyclic (Crounse and Stengle, 1959; Oliver, 1967a, 1967b; Dry, 1926; Chase et al., 1951; Chase, 1954; Kligman, 1959). After a period of active growth in anagen, matrix cells cease to divide, and the lower follicle regresses during catagen (Straile et al., 1961). When the regression is completed, the follicle enters telogen, a resting phase that lasts for several months. The matrix cells then resume proliferation and produce a new hair bulb, thus reentering anagen and completing a hair cycle.

It has generally been assumed that matrix cells, through their interactions with the dermal papilla, play a central role in follicular growth and differentiation (Kligman, 1959; Van Scott et al., 1963; Pinkus, 1978). Given the important

role attributed to the matrix, it is puzzling why a complete hair follicle can be regenerated after the matrix-containing hair bulb is surgically removed (Oliver, 1966, 1967a, 1967b). In addition, the controlling mechanism responsible for the cyclic growth of the hair has been enigmatic.

A key issue that must be addressed when one considers the homeostasis and differentiation of any self-renewing tissue such as the hair follicle is the location of its stem cells. Based on previous studies of stem cells of the hemopoietic system and of several stratified squamous and simple epithelia, we know that stem cells possess many of the following properties (Gilbert and Lajtha, 1955; Potten et al., 1979; Leblond, 1981; Lavker and Sun, 1982, 1983; Buick and Pollak, 1984; Wright and Alison, 1984; Schermer et al., 1986; Cotsarelis et al., 1989): they are relatively undifferentiated, both ultrastructurally and biochemically; they have a large proliferative potential and are responsible for the long-term maintenance and regeneration of the tissue; they are normally slow cycling, presumably to conserve their proliferative potential and to minimize DNA errors that could occur during replication; they can be stimulated to proliferate in response to wounding and to certain growth stimuli; they are often located in close proximity to a population of rapidly proliferating cells corresponding to the transient amplifying (TA) cells in the scheme of stem cell→TA cell→terminally differentiated cell; and finally, they are usually found in a well-protected, highly vascularized and innervated area.

One of the most distinguishing features of stem cells is their slow-cycling nature (Wright and Alison, 1984). This means that a single pulse of tritiated thymidine ([³H]TdR) will not label stem cells; rather, their labeling requires repeated administration of [³H]TdR for a prolonged period. Once labeled, cells that cycle slowly will retain the isotope for an extended period of time and thus can be identified as label-retaining cells (LRCs) (see Bickenbach, 1981; Morris et al., 1985).

Using this approach, we have identified a discrete population of mouse hair follicle cells that are slow cycling but can be induced into the proliferative pool in response to hyperproliferative stimuli, e.g., O-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promotor. In addition, they possess a relatively undifferentiated cytoplasm and are well protected (they always remain intact and are left behind after hair plucking). The location of these cells was unexpected as they were not found in the matrix area of the bulb, where follicular stem cells are currently thought to reside. Instead, the cells were identified in a specific area of the outer root sheath called the "Wulst" or bulge (Unna, 1876; Stohr, 1903–1904; Pinkus, 1958; Madsen, 1964), the attachment site of the arrector pili muscle. This area is below the opening of the sebaceous gland, and it marks the lower end of the "permanent" portion of the hair follicle; keratinocytes below it degenerate during catagen and telogen phases of the hair cycle (Montagna, 1962). In embryonic hair, the bulge is a prominent structure sometimes even larger than the hair bulb (Pinkus, 1958). However, it
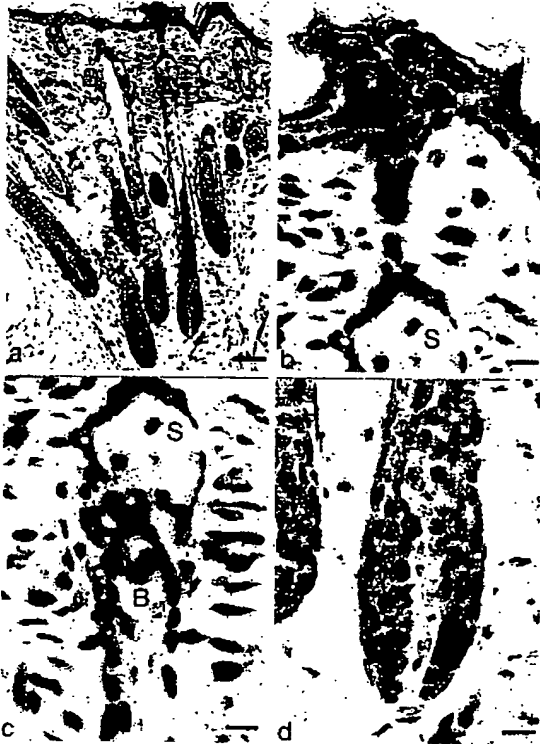
Figure 1. Long-Term L? .eling of Neonatal Mouse Skin with [³H]TdR

Newborn SENCAR mice were injected subcutaneously with [³H]TdR (5 µCi per gram of body weight) twice daily for the first 7 days of life, and portions of flank skin were prepared for 3 µm plastic sections followed by autoradiography.

(a) Low magnification survey picture showing the epidermis (E), sebaceous gland (S), and hair matrix (M).

(b–d) High magnification views of the epidermis, sebaceous gland, and matrix, respectively. Note that almost all nuclei are labeled, including those in the hair matrix (M).

Calibration bar in (a) = 100 µm. Calibration bars in (b–d) = 13 µm.
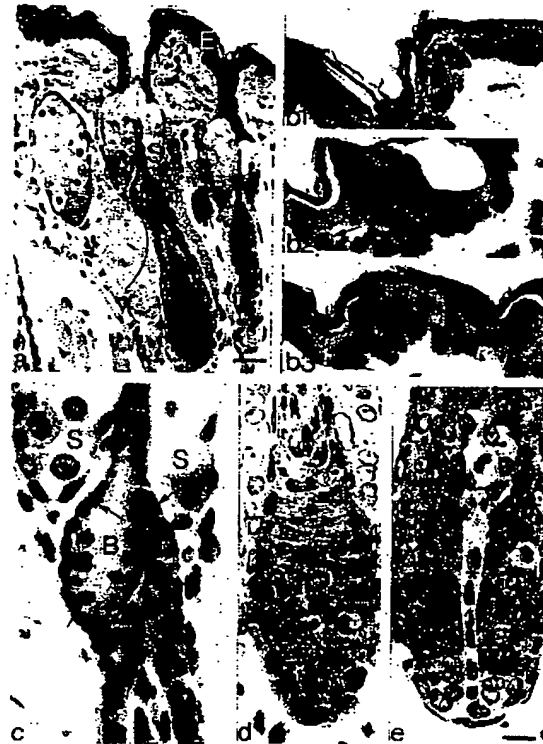

Figure 2. Identification of Label-Retaining Cells in the Mouse Pilosebaceous Unit

Mice that were long-term labeled as described in Figure 1 were chased for 4 weeks and sacrificed, and skin sections were prepared for autoradiography. A survey picture is presented in (a), portions of which are shown at a higher magnification in (b), (c), and (d). The epidermis from three different areas is shown in (b1–b3); note occasional nuclei containing ³H labeling (arrows). In (c) the bulge (B) area of a hair follicle (also labeled as B in [a]) is shown, situated next to a well-developed sebaceous gland (S) with foamy sebocytes. Note that in the bulge area many cells contain labeled nuclei; an interesting feature of many of these bulge cells (arrows) is their irregularly shaped nuclei. In (d) is shown a tangential cut of the bulb area from the same follicle whose bulge area is shown in (c). Note the lack of LRCs in the matrix (M), although many mitotic figures (arrows) are evident. A vertical section of another bulb illustrating the absence of LRCs is shown in (e); the bulge area of this follicle contains LRCs (not shown). The asterisk denotes the dermal papilla. Calibration bar in (a) = 40 µm. Calibration bar in (e) = 13 µm; micrographs in (b–d) are at the same magnification.

is relatively inconspicuous in routine paraffin sections of adult follicles. Consequently, the bulge has attracted so little attention in the past that it is rarely mentioned in histology textbooks (Cormack, 1987; Stenn, 1988). The realization that hair follicle stem cells may reside in the bulge area provides new insights into how the hair cycle may be regulated and the possible involvement of the hair follicles in skin carcinogenesis.

Results and Discussion

Identification of Slow-Cycling Cells in Mouse Hair Follicles

To label the normally slow-cycling follicular stem cells, we took advantage of the fact that in mice the formation of hair follicles occurs predominantly during the first 7–10 days of postnatal life (Dry, 1926; Wolbach, 1951). During this period, the follicular cell population expands and most cells readily incorporate [³H]TdR. Twice daily subcutaneous injections of [³H]TdR into newborn SENCAR mice

over the first 7 days of life resulted in the labeling of almost 100% of nuclei in mouse epidermis, hair follicles, and sebaceous glands as well as fibroblasts and endothelial cells, as revealed by autoradiography of 3 µm plastic-embedded tissue sections (Figure 1). After the label was chased from these animals for an additional 4 weeks, no LRCs were identified in the matrix area of any of the hair follicles (Figures 2d and 2e), indicating that the matrix does not contain slow-cycling cells. Indeed, as early as 1 week after cessation of [³H]TdR labeling (1 week chase), the matrix cells were devoid of label (data not shown), suggesting that essentially 100% of matrix cells are involved in rapid proliferation. This supports an earlier suggestion that the growth fraction of hair matrix cells approaches
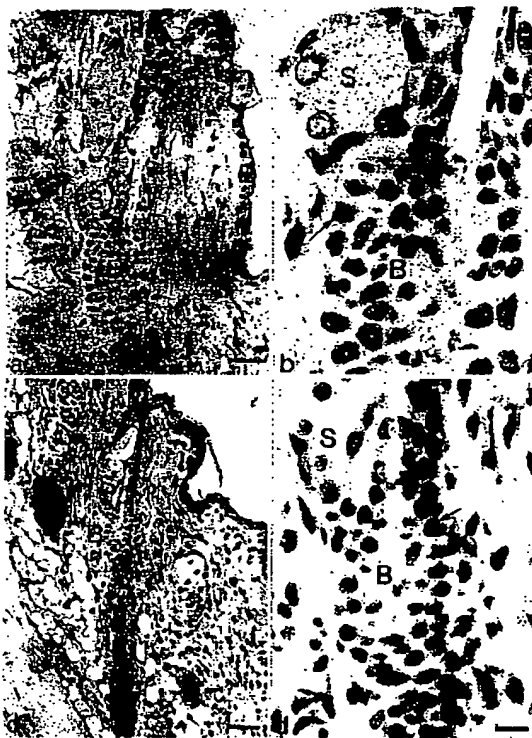
Figure 3. Two Other Examples of the Bulge Area Containing LRCs

Low magnification survey pictures of two different fields are shown in (a) and (c). The areas of these two pictures containing the bulge (B) and parts of the sebaceous gland (S) are shown at a higher magnification in (b) and (d). Note the irregularly shaped nuclei in some of the bulge cells (arrows) and the absence of LRCs in sebaceous glands (S). Calibration bar in (a) = 40 µm. Calibration bar in (c) = 66 µm. Calibration bar in (d) = 13 µm; the micrograph in (b) is at the same magnification.

Figure 4. Identification of LRCs in Adult SENCAR Mouse Epidermis and Hair Follicles

Adult mice were continuously labeled for 2 weeks using implanted pumps. To induce the slow-cycling cells into a proliferative phase, the skin was treated topically with TPA once daily for the first 4 days (see Experimental Procedures). After the labeling was completed, the label was chased for 4 weeks, and skin sections were prepared for autoradiography. In (a) and (b) several LRCs (arrows) in the epidermis are illustrated. Low magnification survey pictures in (c) and (e) show two different fields with their bulge (B) areas magnified in (d) and (f), respectively. Note the LRCs (arrows) in the bulge (B) areas and their absence in sebaceous glands (S). Calibration bar in (f) = 13 µm; micrographs in (a), (b), and (d) are at the same magnification. Calibration bar in (c) = 100 µm. Calibration bar in (e) = 66 µm.

unity (Weinstein, 1979). Unexpectedly, groups of LRCs can be seen in the midfollicle, in an area of the outer root sheath that can be identified morphologically as the bulge (Figures 2c and 3). These bulges usually lie immediately below, and at times appear to be pressed against, the sebaceous glands, which did not contain any LRCs. A striking morphological feature of many of these bulge cells is their irregularly shaped nuclei (Figures 2c, 3b, and 3d). This feature has previously been described in the bulge cells of human hair follicles (Madsen, 1964).

Similar results were obtained when we repeated these experiments using adult SENCAR mice implanted with Alzet osmotic minipumps, which continuously delivered a total of 20 µCi of [³H]TdR per day for 2 weeks. After a 4 week chase period, LRCs were again found exclusively in the bulge region; no LRCs were found in other areas of the follicle, including the matrix and the sebaceous glands (data not shown). Although these results confirmed the neonatal data, it should be noted that in these experiments we found fewer follicles containing LRCs in their bulges, and each LRC contained, in general, fewer grains per nucleus. Most likely this is because the bulge regions

of only a few adult hair follicles were replicating during the 2 weeks of continuous labeling. To test this hypothesis, we painted mouse skin during the first 4 days of the labeling period with TPA, a potent tumor promotor known to stimulate DNA synthesis in both the epidermis and hair follicle (Schweizer and Marks, 1977; Bhisey et al., 1982, Ogawa and Hattori, 1983). This resulted in a markedly increased number of LRC-containing bulges and a significantly higher number of silver grains per labeled nucleus (Figures 4c–4f). This indicates that the normally slow-cycling cells within the adult bulges (as well as other cells) can be stimulated to proliferate. Significantly, once the external stimulation was removed, the bulge cells were apparently the only ones that returned to their previously slow-cycling state, as they were the only cells observed within the pilosebaceous unit to retain their label for a long period of time. Similar to what we found in the skin of newborn SENCAR mice (Figure 2b), only a few LRCs could be de-
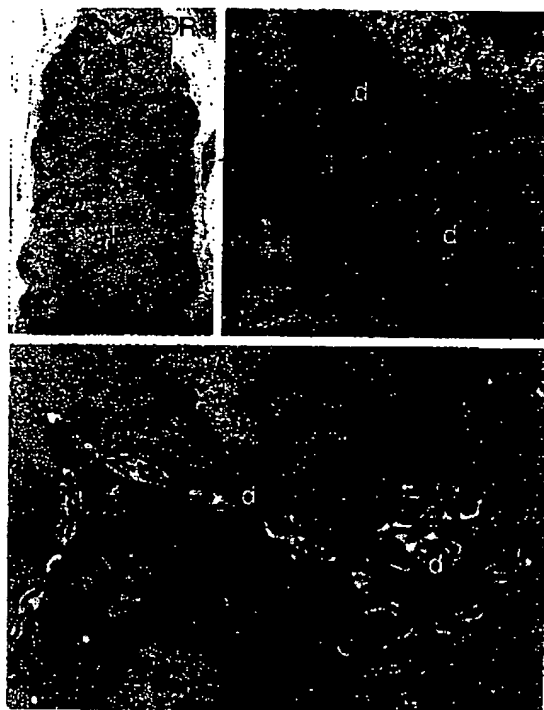
Figure 5. Ultrastructural Characterization of the Bulge Cells

(a) Low magnification survey picture of a bulge (B) budding out from the outer root sheath (ORS). Calibration bar = 5 μm.

(b) Two outer root sheath basal cells showing prominent tonofilament bundles (t) attached to desmosomes (d). N, nucleus. Calibration bar = 0.5 μm.

(c) The bulge cells with numerous microvilli (m) projecting into the intercellular space and a "primitive" cytoplasm filled with ribosomes. d, desmosome; N, nucleus. Calibration bar = 0.5 μm.

tected in the basal layer of normal adult epidermis; however, epidermal LRCs were observed with greater frequency in the TPA-treated skin (Figures 4a and 4b).

These results indicate that a population of LRCs or slow-cycling cells exists exclusively in the bulge area of the hair follicle. To assess the state of differentiation in these cells, we examined them by transmission electron microscopy. These cells are characterized by a cytoplasm filled with ribosomes and relatively devoid of keratin filament bundles (Figure 5c); these features are typical of relatively undifferentiated or "primitive" cells. Ultrastructurally, these bulge cells are extremely similar to the unserrated basal cells of the deep rete ridges of monkey palm epidermis. These unserrated cells were also found to be slow cycling and were identified as the putative stem cells of the palm and sole epithelia (Lavker and Sun, 1982, 1983).

Although these data alone do not prove conclusively that the LRCs of the bulge area are stem cells, a critical reevaluation of the literature revealed additional evidence, previously thought to be paradoxical, that lends strong support to the notion that hair follicle stem cells reside in the bulge. For example, Montagna and Chase (1956) found that X-irradiation can destroy the hair matrix, but

cells in the outer root sheath can regenerate a complete hair bulb. Based on this, Montagna (1962) suggested that "the seed, or germinative source of each generation of hair follicles, must reside in the outer root sheath and not in the bulb." Consistent data were obtained by Oliver (1966, 1967a, 1967b) and Ibrahim and Wright (1982A), who showed that the lower half of rat (vibrissa) hair follicles can be surgically removed, and a new hair bulb can regenerate in response to the implantation of a new dermal papilla. This result strongly suggests that hair follicular stem cells are located in the upper half of the follicle, which includes the region of the bulge. Finally, surgical removal of human axillary hair follicles up to a level near the sebaceous gland did not prevent the regeneration of new hair follicles (Inaba et al., 1979). This again suggests that hair follicle stem cells are located in the upper half of the follicle in an area immediately below the sebaceous gland opening, i.e., the region of the bulge.

The bulge structure is by no means unique to the hair follicles of the mouse. Madsen (1964) identified outer root sheath bulges in human trunk and neck skin; however, bulges are less conspicuous in the follicles of the scalp. Similar lateral swellings or bulges have also been described in embryonic and adult follicles of the sheep (Auber, 1952).

Advantages of the Bulge As a Site of Hair Follicle Stem Cells

There are several biological advantages for follicular stem cells to be located in the bulge area instead of the lower bulb. First, unlike the hair bulb, which undergoes cyclic degeneration, cells in the bulge area are in the permanent portion of the hair follicle. Situated at the lower end of the permanent portion, bulge cells are in a strategically convenient position to interact, during late telogen, with the upcoming dermal papilla and to send out a new follicular downgrowth during early anagen (see below). Second, basal cells of the bulge form an outgrowth pointing away from the hair shaft (Figures 2a and 4c) and are therefore safeguarded against accidental loss due to plucking. The bulb area, however, is vulnerable to damage by plucking (Bassukas and Hornstein, 1989). An analogous situation exists in limbal epithelium (site of corneal epithelial stem cells), which adheres tightly to the underlying stroma and is therefore extremely resistant to shearing. In contrast, corneal epithelium is easily torn from its stromal base (Schermer et al., 1986; Cotsarelis et al., 1989). Finally, the bulge is a reasonably vascularized area so that cells in this area are well nourished (Ellis and Moretti, 1959; Montagna, 1962).

Hair Cycle: The Bulge Activation Hypothesis

Although the histological changes occurring during the growing (anagen), regressing (catagen), and resting (telogen) phases of the hair cycle have been described in detail, the mechanistic basis underlying the cessation and reactivation of follicular growth has remained obscure (Johnson, 1965; Ebling, 1976).

The recognition that hair follicle stem cells may reside in the bulge area, coupled with the current concept of

a. ANAGEN VI   b. CATAGEN   c. TELOGEN
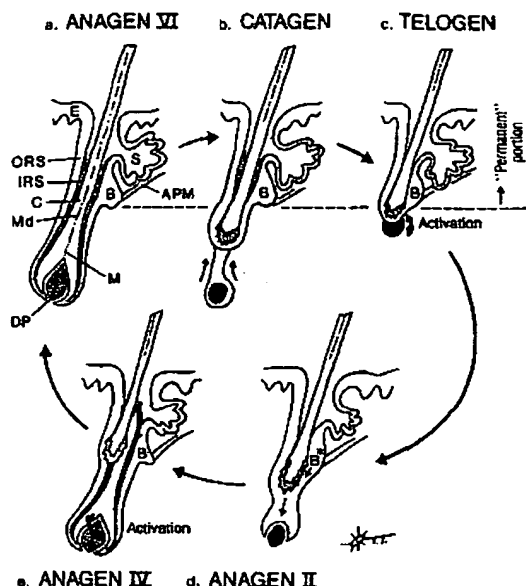
e. ANAGEN IV   d. ANAGEN II

Figure 6. Hair Cycle: The Bulge Activation Hypothesis

Illustrated are different phases of the hair cycle including (a) anagen VI, (b) catagen, (c) telogen, (d) anagen II, and (e) anagen IV. Different structures are labeled, including the arrector pili muscle (APM), bulge (B), cortex (C), dermal papilla (DP), epidermis (E), inner root sheath (IRS), matrix (M), medulla (Md), outer root sheath (ORS), and sebaceous gland (S). B and B* denote quiescent and activated bulge cells, respectively. Follicular structures above the dashed line form the permanent portion of the follicle; keratinocytes below the bulge degenerate during catagen and telogen and are therefore dispensable. This model defines four major elements involved in controlling the cyclic growth of hair follicles: activation of the bulge by dermal papilla (c), activation of mesenchymal papilla by the growing matrix (e), limited proliferative potential of matrix cells as TA cells (a), and upward migration/movement of the dermal papilla (b).

stem cells, led us to propose a model that provides a conceptual framework regarding how the hair cycle may be controlled. This model, shown schematically in Figure 6, takes into account most of the critical events known to occur during various phases of the hair cycle. The salient features of this model are presented below.

Activation of Bulge Stem Cells by the Dermal Papilla during Late Telogen

During late telogen or early anagen, the normally slow-cycling stem cells of the bulge area are activated (designated in Figure 6d as B*) by dermal papilla cells. The mechanism of this activation is unknown but could involve a diffusible dermal papilla–derived growth factor and/or direct cell–cell contact (Sugiyama et al., 1976), which is also known to occur during a comparable stage in embryonic hair formation (Robins and Breathnach, 1969). This activation leads to the proliferation of some cells in the bulge area (the germinal epithelium; Chase, 1954), which forms a downgrowth. As the dermal papilla is pushed away from the bulge by this newly formed epithelial column, the bulge stem cells presumably return to their normally quiescent, slow-cycling state (designated as B in Figure 6) in midanagen. Experiments are in progress to test this hypothesis.

Activation of the Dermal Papilla by the Matrix during Midanagen

The dermal papilla is known to undergo hair cycle–dependent changes in its volume, histological appearance, and basement membrane composition (Montagna, 1962; Puccinelli et al., 1968; Couchman and Gibson, 1985). During most phases of the hair cycle, the dermal papilla appears to be relatively dormant (Moffat, 1968). However, Pierard and de la Brassinne (1975) showed that during anagen stage IV there is a burst of cell proliferation in the dermal papilla. Some of these replicating papillary cells are endothelial cells engaged in angiogenesis, which also occurs in the perifollicular area (Pierard and de la Brassinne, 1975; Sholley and Cotran, 1976). Since matrix (keratinocyte) proliferation clearly precedes vascular proliferation, these results suggest that during midanagen (stage IV), matrix cells are capable of stimulating dermal papilla and other perifollicular mesenchymal cells to undergo proliferation and possibly metabolic activation (Silver and Chase, 1977). In support of this possibility, Stenn et al. (1988) have recently demonstrated angiogenic activity of anagen hair matrix. Possible consequences of this activation include the formation of new blood vessels that would undoubtedly facilitate rapid hair growth (Hockel et al., 1984). In addition, this activation may "loosen up" and thus increase the volume of the dermal papilla. This step may be crucial since Van Scott and Ekel (1958) and Ibrahim and Wright (1982) have shown that the volume of the hair matrix as well as the diameter and length of the resulting hair is strictly proportional to the volume of the dermal papilla.

Matrix Cells Are TA Cells

Since almost 100% of matrix cells are involved in continuous replication, they are most likely TA cells derived from the putative stem cells residing in the distant bulge area. The scheme of stem cells→TA cells→terminally differentiated cells has been described previously in detail (Lajtha, 1979; Potten et al., 1979; Lavker and Sun, 1982, 1983; Buick and Pollak, 1984; Wright and Alison, 1984; Cotsarelis et al., 1989; Hall and Watt, 1989). According to this concept, stem cells possess an unlimited life span (relative to the life span of the animal) and are slow cycling (Wright and Alison, 1984). After each stem cell division, on average one of the two (probably identical) daughter cells leaves the stem cell niche (the bulge area, which presumably contains local environmental factors essential for the maintenance of the cell's "stemness") and becomes a TA cell (the matrix cell; see Schofield, 1983). Since such TA cells, by definition, have only a limited proliferative potential, they eventually become exhausted and undergo terminal differentiation, which is exactly what happens during catagen (Straile et al., 1961). According to this hypothesis, the length of anagen is an intrinsic property of the matrix cells; this may explain why, once started, the length of the growing phase is relatively insensitive to environmental factors (Ebling, 1976).

Upward Movement of Dermal Papilla Is Critically Important for the Activation of Hair Stem Cells

During early catagen, the dermal papilla is condensed and becomes increasingly distant from the regressing matrix. They are still connected, however, by an epithelial

column or sheath (Figure 6b). Later, the dermal papilla is pulled upward, presumably through the contractile activities of the outer root sheath cells and/or the connective sheath cells that wrap around the dermal papilla (for references see De Weert et al., 1982). This upward movement of the dermal papilla apparently plays a critical role in the subsequent activation of cells in the bulge area. A striking example illustrating the importance of this movement is provided by the hairless mouse mutant (Montagna et al., 1952; Chase, 1954). In this animal, the first round of hair growth is normal up to catagen with the formation of an epithelial strand connecting the club hair and dermal papilla (Figure 6b). However, in the mutant this strand fails to shorten and becomes constricted and broken at several places, possibly due to a defective connective tissue sheath. Consequently, the dermal papilla stays down, and no subsequent hair growth occurs, presumably because the bulge cells cannot be activated.

Biological Implications of the Bulge
Stem Cell Model
In Vitro Cultivation of Hair Follicle Cells
Although dermal papilla cells have been successfully cultured and, at least for a limited period of time, retain their ability to induce follicular regeneration (Jahoda et al., 1984; Messenger, 1985; Horne et al., 1986), no one has yet developed a satisfactory method for growing in vitro keratinocytes capable of undergoing (hair shaft–type) follicular differentiation. This may be attributed to two reasons. First, most efforts to date have focused on matrix cells, which, being TA cells with an exceptionally strict dermal papilla dependence (Figure 6), may have a rather limited in vitro life span (Rogers et al., 1987; T.-T. S., unpublished data). Second, efforts have been made to grow the outer root sheath cells mostly using plucked hair as a source (Weterings et al., 1982; Imcke et al., 1987). Since we know that basal cells of the bulge are quite resistant to physical removal and tend to remain behind after hair plucking (data not shown), such cultures are probably devoid of bulge cells. Future experiments designed to grow the bulge cells, with cocultured dermal papilla cells and/or their secreted products, could be rewarding.
Diseases of the Hair Follicle
In the model presented in Figure 6, we have defined four important elements in controlling hair growth: bulge activation, dermal papilla activation, the limited proliferative potential of matrix cells, and the upward migration of the dermal papilla. Defects in any of these elements could result in abnormal hair growth or hair loss, as exemplified by the hairless mouse situation cited earlier. With this as a basis, we believe that a critical reexamination of certain aspects of follicular pathology and cell kinetics, paying special attention to abnormalities in the bulge and dermal papilla during different phases of the hair cycle, could lead to an improved classification and a refined understanding of various hair diseases.
Follicular Origin of Most Experimentally
Induced Skin Carcinomas
Mouse skin has been used extensively as a model system for studying chemical carcinogenesis (Yuspa, 1986). A

carcinogen is usually applied topically onto a shaved area of the skin, and subsequent tumor formation is observed. A previously unexplained outcome of this type of experiment is that tumor formation shows a strict dependence on the hair cycle, with the resting phase (telogen) being most susceptible (Andreasen, 1953; Borum, 1954; Argyris, 1980). For example, Borum (1954) found that of the 55 mice that were treated with 9,10-dimethyl-1,2-benzanthracene during the growing phase of the hair cycle, none (0%) gave rise to papillomas. In contrast, 40 (88%) of the 45 animals treated during the resting phase of the hair cycle developed skin papillomas. Although this implies that the hair follicle plays a central role in skin carcinogenesis, the idea has remained controversial (see, e.g., Giovanella et al., 1970; Iversen and Iversen, 1976). Argyris (1980) summarized the uncertainties in this area by stating that "until more definitive experiments are completed, we should keep an open mind that it is possible that epidermal tumors promoted by TPA or abrasion arise from hair follicle cells."

In this regard, our finding that follicular stem cells may reside in the bulge area is highly significant. This is because outer root sheath cells of the upper follicle, including the bulge cells (Figures 2–4), are known to be more accessible to topically applied skin carcinogens during the resting phase (telogen) of the hair cycle, when the inner root sheath cells, which normally seal the hair canal, are absent (Figure 6c; Wolbach, 1951; Roth, 1967). Specifically, Berenblum et al. (1959) showed that topically applied carcinogens penetrate better and are retained up to 10 times longer in telogen follicles than in anagen follicles. Therefore, follicular stem cells in the telogen phase are effectively exposed to a much higher concentration of the carcinogen for a longer period of time than stem cells of anagen follicles or interfollicular epidermis. The striking correlation between the tumor yield and the enhanced accessibility of the follicular stem cell zone to carcinogens strongly suggests that stem cells of the hair follicle, rather than interfollicular epidermis, are involved in and are largely responsible for experimental tumor formation in mouse skin. There are additional data that lend support to this thesis. First, Ghadially (1961) studied the role of the hair follicle in the origin and evolution of some experimentally produced cutaneous neoplasms. He found that the most common carcinogen-induced epithelial tumor of the skin (the type I keratoacanthoma) arises from the superficial part of the hair follicle. He believes that "the so-called papillomas experimentally induced in mouse by the carcinogens are, in fact, the result of early exfoliation of type I keratoacanthomas and as such are tumors derived from the necks of the hair follicles." Bhisey et al. (1982) also shared the view that most mouse skin tumors are follicle related. Second, Steinmuller (1971) showed in transplantation experiments that papillomas can arise from the epithelial rudiments of carcinogen-exposed hair follicles. Finally, Morris et al. (1986) demonstrated recently that 2% of mouse epidermal basal cells and 4%–5% of infundibulum and outer root sheath cells (but, interestingly, no matrix cells) retain carcinogen for a long period of time and are thus likely to be responsible for tumor formation. Al-

though this result does not differentiate between follicular and interfollicular cells in terms of their relative contributions to tumor formation, it argues against the involvement of matrix cells in skin chemical carcinogenesis.

Taken together, these data strongly suggest that a great majority of the tumors experimentally produced on mouse skin during the resting phase of the hair cycle are derived from follicular stem cells (of the bulge area) instead of the epidermis. Although this does not diminish the importance of studying experimentally induced tumors, it does raise questions regarding the immediate relevance of such data to epidermal tumors. For example, what are the relative contributions of the follicle and interfollicular epidermis in giving rise to various human skin carcinomas? Do human tumors derived from the epidermis behave in the same way as those originating from the hair follicle? These questions are of particular interest because of the possibility that what we have learned about cutaneous tumors by studying chemical carcinogenesis of mouse skin may turn out to be immediately applicable to only follicle-derived tumors.

Pluripotent Stem Cells?

Primarily based on the behavior of epidermal and follicular tumors, Pinkus and Mehregan (1981) hypothesized the existence of a population of skin pluripotent stem cells that can give rise not only to hair follicles, but also to sebaceous gland and the epidermis. This view was also expressed by Chase (1954) who, based mainly on teleological arguments, actually placed such hypothetical pluripotent stem cells in the "upper outer (root) sheath." Our identification of a population of putative stem cells located exclusively in the bulge area (Figures 2–4), which occupies a strategically central position immediately adjacent to the opening of the sebaceous gland (which, interestingly, contains no LRCs, suggesting that its stem cells may exist elsewhere) as well as the epidermis, is consistent with their being the hypothesized pluripotent stem cells. Additional experiments are needed to test this idea and to define the relationship between the bulge cells and the slow-cycling epidermal (stem) cells.

Experimental Procedures

Localization of Label-Retaining Cells

SENCAR mice (Charles River Lab., Boston, MA) were used in all our experiments. To label slow-cycling cells in neonatal mice, animals received subcutaneous injections of 5 μCi of [methyl-³H]TdR (specific activity 82.7 Ci/mmol; New England Nuclear, Boston, MA) per gram of body weight twice daily for the first 7 days of life (5–15 μCi per animal per day, depending on the weight of the mouse). Afterward the mice were sacrificed either immediately (time 0) or at weekly intervals for up to 8 weeks. Pieces of flank skin were excised, fixed in 10% phosphate-buffered formalin, embedded in JB-4 plastic, sectioned at 3 μm, and assayed by autoradiography (Cotsarelis et al., 1989). Since at no time were there more than two grains over a nucleus in control tissues not exposed to [³H]TdR (i.e., background), nuclei with five or more grains were unequivocally labeled and were considered LRCs.

To label slow-cycling cells in adult (4-week-old) mice, two Alzet osmotic minipumps (model 2002, Alza Corp., Palo Alto, CA) were loaded with 200 μCi of [³H]TdR, were implanted intraperitoneally in each SENCAR mouse. Each pump delivered 10 μCi of [³H]TdR (in 10 μl) per day; the total dose is therefore 20 μCi per animal per day. After a continuous labeling of 14 days, pumps were surgically removed and the mice sacrificed either immediately or at weekly intervals for up to 8

weeks. At this dose the animals appeared healthy, with normal skin histology during the entire course of the experiments. Tissues were harvested and processed as described above.

Inducement of Slow-Cycling Cells into a Proliferative Phase

To facilitate detection of LRCs in adult mice, we induced these cells to proliferate (Cotsarelis et al., 1989). Alzet osmotic minipumps were implanted intraperitoneally in adult SENCAR mice as described. During the first 4 days of the 14 day labeling period, 0.01% TPA in petrolatum was applied topically once daily to the right flank. As a control, mice were treated with petrolatum only. The animals were sacrificed and tissue was processed as described.

Histology and Autoradiography

After fixation in formalin, tissues were dehydrated through a graded series of ethyl alcohol and infiltrated overnight in a monomer solution of JB-4 water-soluble plastic media (Polysciences, Fort Washington, PA). Tissues were embedded in JB-4 embedding medium, and 3 μm sections were cut with a glass knife on a Reichert-Jung 2050 Supercut microtome (Cambridge Instruments, Buffalo, NY). Autoradiography was performed as described earlier (Cotsarelis et al., 1989).

Transmission Electron Microscopy

Pieces of neonatal and adult flank skin were fixed with paraformaldehyde–glutaraldehyde and were processed for transmission electron microscopy as previously described (Lavker and Sun, 1982).

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